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Analysis of Common Mutations in the Galactose-1-Phosphate Uridyl Transferase Gene

New Assays to Increase the Sensitivity and Specificity of Newborn Screening for Galactosemia

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Classical galactosemia is a genetic disease caused by mutations in the galactose-1-phosphate uridyl transferase (GALT) gene. Prospective newborn screening for galactosemia is routine and utilizes the universally collected newborn dried blood specimen on filter paper. Screening for galactosemia is achieved through analysis of total galactose (galactose and galactose-1-phosphate) and/or determining the activity of the GALT enzyme. While this approach is effective, environmental factors and the high frequency of the Duarte D2 mutation (N314D) does lead to false positive results. Using DNA derived from the original newborn dried blood specimen and Light Cycler technology a panel of five assays able to detect the four most frequently encountered classical galactosemia alleles (Q188R, S135L, K285N, and L195P) and the N314D Duarte variant mutation are described. The five assays are performed simultaneously using common conditions. Including DNA preparation, set-up, amplification, and analysis the genotype data for all five loci is obtained in less than 2 hours. The assays are easily interpreted and amenable to high-throughput newborn screening. Mutational analysis is useful to reduce false positive results, differentiate D/G mixed heterozygotes from classical galactosemia, and to clearly identify a very high percentage of those affected by classical galactosemia. *(J Mol Diagn 2003, 5:42–47)*

Classical galactosemia (OMIM 320400) is a genetic disease caused by mutations in the galactose-1-phosphate uridyl transferase (GALT) gene. In recent years this gene has been characterized and numerous mutations, including several common mutations, have been identified.[1,2] Prospective screening of newborns for galactosemia is a widely accepted procedure both in the United States and throughout the world. Screening utilizes the Guthrie dried blood specimen collected on filter paper to assay for total galactose (galactose plus glactose-1-phosphate) and/or activity of the GALT enzyme using a modification of the Beutler screening test.[3]

False positive results in newborn screening for galactosemia are frequent and represent a substantial problem for screening programs. A common observation is the adverse affect that environmental factors and sample-handling procedures (practiced at the site of specimen collection or during specimen transport) may have on the GALT assay, resulting in low activity and false positives.[4] The most notable environmental influences are heat and especially humidity. Specimens collected during hot, humid summer months or in climates where such conditions are persistent often present with reduced GALT activity. Also, since the activity of the GALT enzyme in a dried blood specimen deteriorates over time at room temperature, the practice of batching, where dried blood specimens are permitted to accumulate at the hospital before being mailed to the screening laboratory, may also adversely affect GALT activity. Thus, a system of distinguishing specimens representing true cases of galactosemia from such false positive results would be of great benefit in newborn screening programs.

Several disease-causing mutations are commonly encountered in classical galactosemia. The most frequently observed is the Q188R classical mutation which has been reported to account for 54 to 70% of classical galactosemia alleles[5–8] The S135L mutation is the most frequently observed mutation in African-Americans and accounts for approximately 50% of the mutant alleles in this population. The K285N mutation is common in those of eastern European descent and accounts for 8% of the alleles in the general European population. The L195P mutation is observed in ~2.6% of classical galactosemia alleles.[6,9,10] The D2 Duarte variant, caused by the N314D mutation, is present in 5% of the general U.S. population

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and reduces activity of the GALT enzyme by ~25%.[11,12] The Duarte N314D mutation, when paired with a classical galactosemia allele, results in the milder and probably benign D/G phenotype with an approximately 75% reduction in enzyme activity. Biochemical data (total galactose and GALT analysis) generated by a D/G mixed heterozygote will often mimic classical galactosemia in the newborn period, resulting in a false positive result.

With the exception of second-tier screening for the common ΔF508 mutation in newborn screening programs for cystic fibrosis, molecular analysis is not widely used in newborn screening. Complexity, turn-around time, and labor intensity associated with traditional methods of gene characterization such as sequence analysis, allele-specific cleavage, and SSCP analysis, has inhibited the widespread use of molecular analysis in newborn screening programs. It is the experience of this laboratory, that supplementing primary screening using traditional biochemical methods with second-tier mutational analysis on DNA from the original DBS specimen, is a powerful method to reduce false positives and often provides definitive confirmation of true positives. The Light Cycler platform effectively eliminates issues of turnaround time and labor intensity encountered with classical methods of mutation analysis.[13,14] The Light Cycler utilizes rapid air driven thermal cycling and in-line fluorescence analysis to generate melting curves, which are subsequently used to generate melting peaks for genotype assignment.[15–23] A second advantage of this platform is the "closed tube" format where amplification and analysis are performed seamlessly in a common reaction vessel. Unified amplification and analysis simplifies sample tracking while greatly reducing the likelihood of amplicon contamination in the laboratory.

Herein we report a five-mutation panel that we are routinely using as a second-tier screen for the identification of classical galactosemia and D/G mixed heterozygotes in the newborn period. Using Light Cycler technology, the panel assays for the four most frequently encountered GALT mutations (Q188R, S135L, K285N, and L195P) associated with classical galactosemia plus the Duarte N314D mutation. The first four mutations represent at least 70% of classical galactosemia alleles. The Duarte N314D mutation is included because it is frequently paired with a classical allele resulting in the D/G mixed heterozygote. Including DNA isolation and set-up, the results of these five assays are available in less than 2 hours.

Materials and Methods

Specimens and DNA Preparation

Using previously described allele-specific cleavage assays, specimens containing the N314D, Q188R, S135L, and K285N mutations were identified in-house, during the course of routine newborn screening for galactosemia.[7–9] Initially, specimens characterized to contain the K285N mutation were provided by Won Ng of Los Angeles Children's Hospital. Specimens containing the L195P mutation were identified using the assay described in this report and putative positives were confirmed by sequence analysis. DNA was isolated from dried blood specimens as previously described and 80 to 130 ng was used as template in each reaction.[24]

Amplification and Hybridization Probes

Sequences of the human GALT gene (GenBank accession numbers M60091, L46699, L46711, L46708, L46363, and L46704) were the basis for primer and probe design. Primers and probes were obtained from Operon Technology (Alameda, CA). PCR reaction buffers (10X stocks) containing 20 mmol/L $MgCl_2$ (used for the K285N and S135L assays) or 30 mmol/L $MgCl_2$ (used for the N314D, Q188R, and L195P assays) were obtained from Idaho Technology (Salt Lake City, UT). Reactions use 1X PCR buffer, 200 μmol/L dNTPs (Roche, Mannheim, Germany), and 0.6 units Klen taq DNA polymerase (AB Peptides, St. Louis, MO) complexed with TaqStart antibody (Clontech, Palo Alto, CA). Complexes between the polymerase and the antibody were made according to manufacturer's instructions. Primers, probes, the concentration at which each is used, and the number of base pairs (bp) in individual amplicons are listed in Table 1.

Amplification is performed in a Roche Light Cycler (Mannheim, Germany). Reactions are cycled 40 times using three temperature segments. Denaturation is achieved at 94°C holding at that temperature for 0 seconds. Primer annealing is performed at 60°C and holding there for 20 seconds. During amplification, fluorescence is acquired at the end of each primer-annealing segment. Product extension is achieved by ramping from 60°C to 72°C at 2°C/second. During all other segments of the amplification process temperature ramping is performed at 20°C/second. In the assays for N314D, S135L, and K285N, amplification is performed in an asymmetric fashion to enrich for the strand to which the hybridization probes bind (sense strand for S135L and N314D, antisense strand for K285N) while the Q188R and L195P assays are amplified in a symmetric manner. Following amplification, the cycling protocol proceeds seamlessly to melting analysis. Melting curve analysis uses the following conditions: 97°C, 0 seconds, ramping at 2°C/second to 40°C, and subsequently ramping from 40°C to 76°C at 0.1°C/second. Fluorescence is acquired continuously during the downward and upward ramps. Melting curves are constructed from data acquired during the upward ramp from 40°C to 76°C. Melting peaks (see Figure 2) are computationally generated by plotting the −dF/dT of the melting curve against temperature.

Fluorescent-labeled probes are analyzed by spectrophotometry for oligonucleotide and fluorophore concentrations. Probes with fluorophore/oligonucleotide ratios of 0.8 to 1.2 are used.[23] All assays use paired hybridization probes for genotype analysis.[15–23] The paired hybridization probe format utilizes two oligonucleotide probes that hybridize to a selected strand of the amplicon. A detection probe is used which hybridizes to a sequence that includes the site of the mutation while an anchor probe

Table 1. PCR Primers, Hybridization Probes and the Concentration at Which Each Is Utilized

Allele	Forward primer sequence	Conc.*	Reverse primer sequence	Conc.	Anchor probe sequence	Conc.	Detection probe sequence	Conc.	PCR product length
N314D	5′ACTGTAAAA GGGCTCTC TCTCC3′	0.5	5′GCAAGCATTTC GTAGCCAA3′	0.3	5′CGCAGGAGCG GAGGGTAGTAA TGAGCGTGCA-FITC 3′	0.3	5′-LC Red 640 CTGCCAATG GTCCCAGTT GG-PO4 3′	0.14	171 bp
Q188R	5′CTTTTGGC TAACAGAGC TCCG 3′	0.5	5′TTCCCATGTCC ACAGTGCTGG 3′	0.5	5′GCCAAGAAAC CCACTGGAGCC CCT-FITC 3′	0.2	5′-LC Red 640 ACACCCTTA CCCGGCAGT G-PO4 3′	0.13	160 bp
S135L	5′CACAGCCAA GCCCTACC TCTC 3′	0.25	5′ACCTCACAAAC CTGCACCCAA 3′	0.125	5′CGTTACATCCA ACCAGGGGT-FITC 3′	0.1	5′-LC Red 640 GAAGCACAT GACCTTACT GGGTGGTGA CGG-PO4 3′	0.2	190 bp
K285N	5′GCTGAGAG TCAGGCTC TGATTCC 3′	0.125	5′CCAGAAATGG TGTTGGGGCT 3′	0.25	5′-LC Red 640 CTTTGAGACGTC CTTTCCCTACTC CATG-PO4 3′	0.2	5′GCTCTTGA CCAATTATAC AAC-FITC 3′	0.1	155 bp
L195P	5′GAGGCTTG GAGGTA AAGGAC 3′	0.5	5′TCCATTAGCAG GGGCTCTCC 3′	0.5	5′-LC Red 640 TGCCCAGCGTG AGGAGCGATCT CAGCAG-PO4 3′	0.2	5′CAGCAGTT TCCCGCCAG ATA-FITC 3′	0.1	149 bp

*Conc., concentration.
All concentrations are given in micromolar and represent the final concentration in the reaction cocktail.

hybridizes to an adjacent sequence. The organization of the anchor and detection probes for each assay are shown in Figure 1. In each assay one probe is labeled 3′ with FITC while the other probe is labeled 5′ with LC-red 640 and 3′ phosphorylated. When the probes hybridize to their targets, the fluorescent moieties are brought into proximity allowing resonance energy transfer. In these assays the detection probes match the mutant allele and subsequently have a one bp mismatch to the wild-type allele.

Results

Figure 2,A–E displays melting peak profiles for the N314D, Q188R, S135L, K285N, and L195P assays, respectively. The N314D, Q188R, and S135L assays (Figure 2, A–C) display analysis of specimens that are homozygous wild-type, homozygous mutant, heterozygous, and a no amplification control. Assays for K285N and L195P (Figure 2, D–E) show the analysis of specimens that are homozygous wild-type, heterozygous, and no amplification control. Specimens homozygous for K285N or L195P are rare and were not available for this study. In all cases, the mutant allele is represented by the high temperature melting peak (perfect match with the detection probe) while the wild-type allele is the low temperature melting peak (one bp mismatch hybrid with the detection probe). Melting peaks are well separated facilitating genotype assignment for each loci. The ΔTm separating the wild-type and mutant peaks ranges from 6.5°C to 10.6°C and are shown in Table 2.

To determine the observed frequency of each of the classical galactosemia mutations, as well as the Duarte mutation, we reviewed all presumptive positive screens that resulted in second-tier molecular screening during a

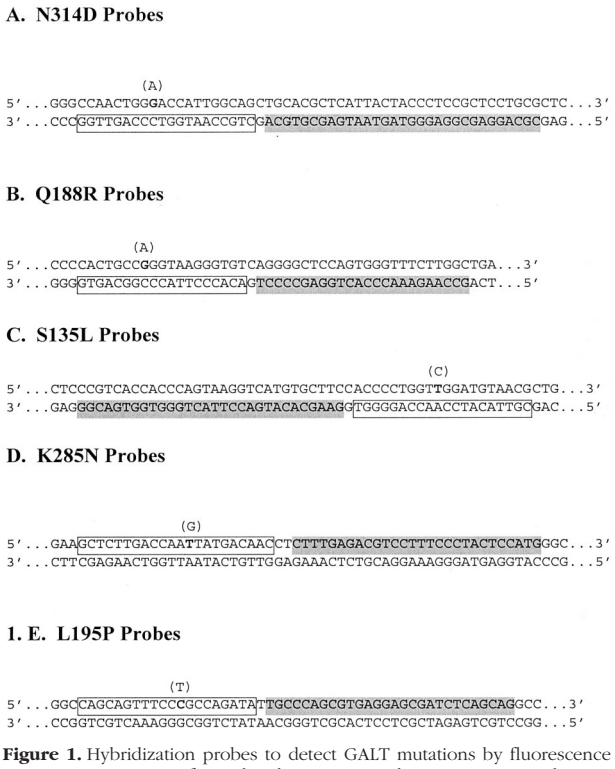

Figure 1. Hybridization probes to detect GALT mutations by fluorescence resonance energy transfer and melting curve analysis. Detection probes are framed while anchor probes are highlighted. All detection probes match the mutant sequence. The mutated nucleotide is shown in **bold font** on the sense strand and the wild-type nucleotide is shown in **parenthesis** above the mutated nucleotide. The detection probes for the N314D and Q188R assays are labeled 5′ with LC red 640 and 3′ phosphorylated, while the detection probes for S135L, K285N, and L195P are labeled 3′ with FITC. Anchor probes for the N314D and Q188R assays are labeled 3′ with FITC, while the anchor probes for S135L, K285N, and L195P are labeled 5′ with LC red 640 and 3′ phosphorylated.

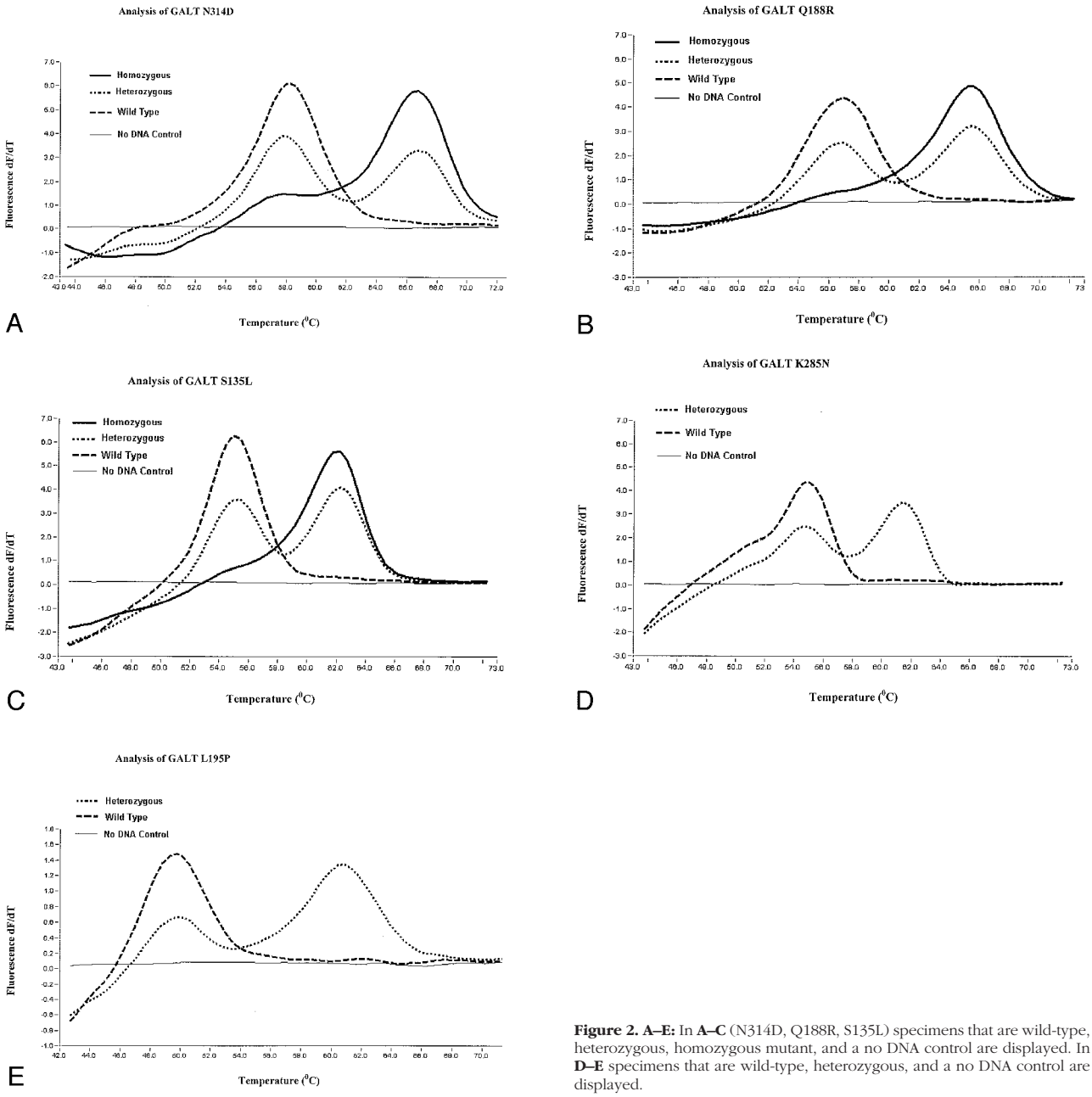

Figure 2. A–E: In **A–C** (N314D, Q188R, S135L) specimens that are wild-type, heterozygous, homozygous mutant, and a no DNA control are displayed. In **D–E** specimens that are wild-type, heterozygous, and a no DNA control are displayed.

seven-month period between January 1, 2002 and July 31, 2002. During this period 150,434 newborns were screened; 266 second-tier molecular analyses were performed and 76 classical mutant alleles were detected. A total of 77.6% were Q188R (59 of 76); 14.5% were S135L (11 of 76); 5.3% were K285N (4 of 76); and 2.6% were L195P (2 of 76). Also, during this period three cases of classical galactosemia were detected including two Q188R homozygotes and one a Q188R/L195P mixed heterozygote. A total of 124 N314D alleles were detected including 26 D/G mixed heterozygotes. These results are close to the mutation frequencies that would be expected in the population screened.

Discussion

Screening for galactosemia is practiced by 49 states in the United States.[25] and extensively worldwide. Biochemical methods used to identify potential cases of galactosemia (analysis of total galactose and/or GALT assays) are sound practices and the concept of primary molecular screening is unlikely owing to the limits of current technology, private mutations within individual pedigrees, and the problem of detecting normal carriers. Screening laboratories are in a difficult position because a false negative result, or any significant delay in detection of a true positive result, may cause serious medical

Table 2. Temperatures at Which Melting Peaks Are Observed for Mutant and Wild Type Form of the Gene

Allele	Wild-type Tm	Mutant Tm	ΔTm
N314D	57.8	66.2	8.4
Q188R	56.8	65.5	8.7
S135L	55.1	62.1	7.0
K285N	54.9	61.4	6.5
L195P	49.9	60.6	10.7

All temperatures in degrees Celsius.

consequences or even death. Thus, to prevent false negative results, screening laboratories set cut-off levels that often result in relatively large numbers of false positives. These generate requests for repeat specimens with the accompanying message of the possibility of disease, which in turn may lead to parental anxiety, mistrust of the screening process, possible unnecessary medical procedures, and significantly increased follow-up costs.

In screening for galactosemia, false positives generally occur as a consequence of either environmental factors affecting specimen performance in the GALT assay, mixed heterozygosity between a classical allele and the Duarte N314D variant, or the presence of normal carriers of a classical allele or Duarte homozygotes. The environmental factors also cause specimens to perform poorly in other enzyme assays. Indeed, it is frequently observed that specimens having low GALT activity owing to environmental factors also have low glucose-6-phosphate dehydrogenase (G6PD) activity for the same reason. A second common reason for "noise" in galactosemia screening is the prevalent Duarte N314D mutation. The Duarte N314D mutation is found in 5% of the general U.S. population and when paired with a classical galactosemia mutation results in the so-called D/G mixed heterozygote, which is probably benign but is often treated with a low galactose-containing diet during infancy. D/G mixed heterozygotes may effectively mimic classical galactosemia in the biochemical assays used in newborn screening. Performing analysis for the Duarte N314D variant, effectively excludes the possibility that a D/G mixed heterozygotes could be erroneously reported as classical galactosemia. The silent mutation (L218L), termed the Los Angeles or D1 Duarte variant, is not associated with reduced GALT activity and as such we make no attempt to identify it.

The remaining mutations in the panel (Q188R, S135L, K285N, and L195P) represent the four most frequently encountered classical mutations.[6] Using these four mutations, many cases of classical galactosemia are clearly identified sooner than would otherwise be possible. Early identification is beneficial in order to avoid the *Escherichia coli* sepsis or hyperbilirubinemia that often presents during the first weeks of life.

Gene level analysis has been historically under-utilized by newborn screening programs. This is due largely to newborn screening's deep roots in biochemical analysis, and issues of throughput and complexity associated with traditional methods of detecting gene level defects. Molecular methods saw their first widespread use in newborn screening for cystic fibrosis during the late 1980s. Screening for cystic fibrosis is generally performed through quantification of circulating immunoreactive trypsinogen (IRT). IRT analysis alone, however, results in a high false positive rate. A two-tiered approach, where specimens having elevated IRT are subsequently assayed for the common CFTR mutation, ΔF508, greatly reduces false positives and is routinely used by laboratories screening newborns for cystic fibrosis.[26–29]

There are numerous metabolic disorders that are components of comprehensive newborn screening programs whose detection will also benefit from analysis of common mutations. Examples include: medium chain acyl-CoA dehydrogenase (MCAD) deficiency, long chain 3-hydroxy acyl-CoA dehydrogenase (LCHAD) deficiency, propionic academia, sickle cell disease, biotinidase deficiency, congenital adrenal hyperplasia, and G6PD deficiency.[30–35] Genetic disorders such as sickle cell disease and biotinidase deficiency are traditional components of newborn screening, but others, such as MCAD deficiency and LCHAD deficiency, are recent arrivals owing to the expanded use of tandem mass spectrometry.[36] The ability to readily detect informative genetic aberrations provides an opportunity for newborn screening programs to deliver valuable information to health care providers while simultaneously increasing the specificity and sensitivity of their programs. In our laboratory, the addition of the described assays to routine screening for galactosemia has reduced the number of false positives, reduced the number of repeat requests, and helped identify cases of classical galactosemia sooner. With the expanding breadth of newborn screening, it is likely that analysis of common mutations will prove beneficial in screening for other disorders.

From a practical point, it is anticipated that it may prove difficult to establish routine second-tier molecular testing in most existing newborn screening laboratories. These laboratories generally do not have the necessary experience or expertise for this level of analysis and are not likely to acquire it in the near future. A sensible approach would be to have a few regional or national reference laboratories to which other screening laboratories could courier the newborn specimens for this second-tier screening.

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